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70 S Monosome Accumulation and *In Vitro* Initiation Complex Formation by *Escherichia coli* Ribosomes at 5 C.

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Running Head: 70 S Monosomes and Initiation Complex Formation at 5 C

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ABSTRACT

The inhibition of translation which is observed after shifting Escherichia coli to low temperature was investigated. 70 S ribosomes which were isolated from E. coli 8 hours after a shift to 5 C synthesized protein in the absence of added mRNA (i.e., endogenous protein synthesis by 70 S monosomes) at a rate which was three times greater than the rate of endogenous protein synthesis by 70 S ribosomes which were isolated at the time of the shift to 5 C. Calculations based on the rates of endogenous protein synthesis and polyphenylalanine synthesis indicate that 70 S monosomes comprise only 0.1% of the total E. coli 70 S ribosome population after 8 hours at 5 C. Experiments designed to test initiation complex formation on ApUpG or formaldehyde treated MS-2 viral RNA demonstrated that, although the rate of formation of 30 S initiation complexes was not inhibited, the rate of formation of active 70 S initiation complexes, able to react with puromycin, was inhibited to a great extent at 5 C. A model depicting the effects of low temperature on the E. coli translation system is proposed.

INTRODUCTION

The minimum temperature which permits the growth of Escherichia coli is in the vicinity of 7.8 C (25). A number of reports concerning the effects of low temperature (near 0 C) on translation by E. coli in vivo (1,5,6,7,11,12) and poly-U directed in vitro (5,28) protein synthesizing systems have linked the inability of this organism to grow to the inability to synthesize protein at temperatures below 7.8 C. Das and Goldstein (7) reported that after shifting E. coli from 37 to 0 C, protein synthesis proceeded at a slowly decreasing rate for a period of 4 hours, while RNA synthesis continued at a linear rate over the same period. They inferred from this and other data that initiation of translation was blocked at 0 C, and, therefore, polypeptide elongation could continue only until the ribosomes ran off the mRNA. In addition, it has been demonstrated that incubation of E. coli at temperatures below 8 C results in polysome runoff (5-7,8,11) and the accumulation of 70 S ribosomes (5,6,7). Anderson (1) investigated the kinetics of B-galactosidase synthesis by E. coli at 5 C and concluded that transfer to 5 C resulted in either a partial inhibition of initiation of lac translation or intracistronic polarity (due to increased rates of endonucleolytic cleavages) for mRNAs coding for large polypeptides.

In a recent study, we (5) investigated the effects of low temperature on in vivo and natural mRNA directed in vitro protein synthesis by E. coli and Pseudomonas fluorescens. These studies demonstrated that initiation of translation proceeded at predictable rates in P. fluorescens, but was blocked in E. coli at low temperature, resulting in polysome runoff and the accumulation of 70 S ribosomal particles. A portion of the 70 S ribosome population which accumulated in E. coli at 5 C were shown to be 70 S monosomes (i.e., 70 S ribosomes with associated mRNA). Based on our data and on the data of others (1,6,7,11,12,28), we postulated that these 70 S

monosomes resulted from an inhibition of initiation of translation at 5 C, and further, that they comprised a minor fraction of the 70 S ribosome population in 5 C incubated E. coli.

We report here the results of experiments designed to determine to what degree 70 S monosomes accumulate in E. coli at 5 C. In addition, in vitro experiments which test specific events involved in initiation complex formation at 5 C are reported and demonstrate that the low temperature inhibition of initiation of translation resides at the level of 70 S initiation complex formation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Escherichia coli K-12 streptomycin sensitive, β-galactosidase and permease constitutive) obtained from Dr. C. Hurwitz, Veterans Administration Hospital, Albany, NY, was grown and maintained as described previously (5).

Preparation of ribosomes, initiation factors, N-formyl-(^{3}H)-methionyl-tRNA, and formaldehyde treated MS-2 RNA.

S-30 cell extracts were prepared by centrifugation of E. coli lysates at 30,000 x g as described previously (5). Crude ribosomes were prepared by centrifugation of the S-30 extracts at 159,000 x g for 2 hours. The resulting supernatant (S-100) was dialyzed against buffer A (10 mM Tris-HCl pH 7.6, 3.5 mM magnesium acetate, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol) and stored in liquid nitrogen. The ribosomes were washed twice with buffer B (10 mM Tris-HCl pH 7.6, 5 mM magnesium acetate, 1 M NH_4Cl, and 6 mM 2-mercaptoethanol), resuspended in and dialyzed against buffer A, centrifuged at 30,000 x g for 20 minutes, and stored in liquid nitrogen.

For the preparation of ribosomal subunits, the crude ribosomal pellet was resuspended in buffer C (similar to buffer A, but containing 1 mM magnesium acetate), layered on 15-30% sucrose gradients (prepared in buffer C), and spun at 27,000 rpm for 18 hours in a Spinco SW 27 Ti rotor at 0 C. The 30 and 50 S peaks were collected and pelleted as described previously (5) and stored in liquid nitrogen.

Crude initiation factor preparations were obtained from the 1 M NH_4Cl ribosomal wash. The washes were combined, dialyzed against buffer A, concentrated by lyophylization, and stored in liquid nitrogen.

N-formyl-(^{3}H)-methionyl-$tRNA_f$ was prepared by incubation of 10 A_{260} units of tRNA (N-formylmethionine specific), 2 μ moles Tris ATP, 5 μ moles phosphoenol pyruvate, 100 μg pyruvate kinase, 180 n moles (^{3}H)-methionine (15 Ci/mmole),

150 μg leucovorin, and 0.3 ml of S-100 in 1.5 mls of buffer (which contained 20 mM Tris-HCl pH 7.6, 8 mM magnesium acetate, 72 mM NH_4Cl, and 6 mM 2-mercaptoethanol) for 15 minutes at 37 C. The tRNA was isolated from the reaction mixture by phenol extraction, precipitated twice with ethanol, dialyzed against 0.1 mM EDTA, and stored at -20 C.

MS-2 phage RNA was treated with formaldehyde as previously reported (5).

Millipore filter assay for initiation complex formation. The binding of N-formyl-(^{3}H)-methionyl-$tRNA_f$ to ribosomes was determined by the filter assay described by Nirenberg and Leder (21). Reaction mixtures (50 μl), which contained 1 A_{260} unit of ribosomes or 0.5 A_{260} units of 30 S subunits, 50 pmoles of N-formyl-(^{3}H)-methionyl-$tRNA_f$, 0.2 μmoles Tris-GTP, 150 μg crude initiation factor preparation, 0.05 A_{260} units adenylyl (3→5') uridylyl(3→5') guanosine (ApUpG) or 20 μg MS-2 RNA, 2% glycerol (w/v), 50 mM Tris-HCl pH 7.6, 150 mM NH_4Cl, 3.5 mM magnesium acetate, and 6 mM 2-mercaptoethanol were incubated at 37 or 5 C. The reactions were stopped at the appropriate times by the addition of 2 mls of ice cold buffer A and were subsequently filtered on nitrocellulose filters (0.45 μm pore size, Millipore Corp., Bedford, MA). The filters were washed twice with ice cold buffer A and dried at 90 C in scintillation vials. Six mls of a toluene based scintillation cocktail (Omnifluor, New England Nuclear, Boston MA) was added to each vial and the radioactivity determined by an Intertechnique SL-30 liquid scintillation counter at about 35% efficiency.

Assay for N-formyl-(^{3}H)-methionyl-puromycin. The reaction mixtures described above were incubated at 37 or 5 C for 15 minutes and then chilled on ice for 2 minutes. Puromycin (50 μg) was then added and the reaction mixtures were returned to the test temperatures. At various times samples (8 μl) were rapidly added to 1 ml of 0.1 M sodium acetate, pH 5.5, and N-formylmethionyl-puromycin was extracted by ethylacetate as described by Leder and Bursztyn (19).

Analysis of initiation complex formation by sucrose gradient centrifugation.

Reaction mixtures for initiation complex formation (100 µl) were incubated at 37 or 5 C for the appropriate times (indicated in the text) and then fixed with an equal volume of 2% glutaraldehyde in buffer A (27). The samples were then layered on 15 to 30% sucrose gradients (made up in buffer A) and centrifuged at 40,000 rpm for 6 hours at 0 C in a SW 41 rotor. The bottoms of the tubes were pierced, the contents of the tubes pumped through a flow cell, and absorbance monitored at 260 nm by a Gilford 2000 spectrophotometer. Fractions (6 drops) were collected in vials, 6 mls of Triton X-100 Omnifluor mixture (1:2) were added, and the radioactivity determined as described above (20% counting efficiency). Sedimentation values were approximated by the method of McEwen (20).

Isolation and in vitro testing of 70 S particles. *E. coli* was grown to mid-log phase at 37 C in 1.5 liters of nutrient broth, rapidly shifted to 5 C, and incubated at 5 C in a reciprocating water bath shaker. This treatment results in polysome runoff within 30 minutes (5). Samples (500 ml) were taken after 15 minutes, 1 hour, and 8 hours of incubation at 5 C, the cells harvested, and crude ribosome and S-100 fractions prepared as described above. The ribosomal pellets from each sample were resuspended in 1 ml of buffer D (identical to buffer A but containing 10 mM rather than 3.5 mM magnesium acetate), layered on 15 to 30% sucrose gradients (prepared in buffer D) and spun at 27,000 rpm for 12 hours at 0 C in a Spinco SW 27 Ti rotor. The bottoms of the tubes were pierced and the contents analyzed as described above. The 70 S peaks from each sample were collected, diluted 3 fold with buffer C, and pelleted by spinning at 40,000 rpm for 5 hours at 0 C in a Spinco Type 42.1 rotor. Each pellet was resuspended in buffer D at a final concentration of 10 mg/ml and tested immediately for the ability to synthesize protein with and without added mRNA (poly-U) at 25 C.

Incorporation of (^{3}H)-amino acids into protein by these 70 S particles was tested at 25 C as reported previously (5). The reaction mixture (50 µl) contained 50 mM Tris-HCl pH 7.6, 60 mM NH Cl, 6.5 mM magnesium acetate, 6 mM 2-mercaptoethanol, 1 mM tris-ATP, 1 mM magnesium-ATP, 0.05 mM Tris-GTP, 5 mM phosphoenol-pyruvate, 5 µg pyruvate kinase, 20 µg tRNA, 0.02 mM (each) ^{3}H-amino acid mix, 0.05 mM each of asparagine, glutamine, cystine, methionine, and tryptophan, 20 µg MS-2 RNA, 60 µg 70 S ribosomes, and 0.2 volumes of S-100. To initiate the reactions 70 S particles plus S-100 were added to the reaction mixture (final ribosome concentration was 1.2 mg/ml). Samples (5 µl), taken at 2 minute intervals, were added to 2 mls of 5% trichloroacetic acid (TCA) and processed as described previously (5). Incorporation of (^{3}H)-phenylalanine into polyphenylalanine was monitored by a similar method with the following modifications: (1) (^{3}H) and (^{1}H)-amino acids were replaced by 0.02 mM (^{1}H)-phenylalanine and 1 µCi (^{3}H)-phenylalanine (15 Ci/mmole); (2) the magnesium acetate concentration was raised to 16 mM, and; (3) MS-2 RNA was replaced by 15 µg of polyuridylic acid (poly-U). Rates of *in vitro* protein synthesis were calculated from the amino acid incorporation vs time plots (i.e., amino acids incorporated into TCA precipitable material/minute/mg 70 particles.

Assay for in vitro incorporation of N-formyl-(^{3}H)-methionine into protein. Reaction mixtures (100 µl), which contained 50 mM Tris-HCl pH 7.6, 60 mM NH_4Cl, 6.5 mM magnesium acetate, 6 mM 2-mercaptoethanol, 1 mM Tris-ATP, 1 mM magnesium-ATP, 0.05 mM Tris-GTP, 5 mM phosphoenol pyruvate, 10 µg pyruvate kinase, 0.05 mM (each) (^{1}H)-amino acids, 40 µg formaldehyde treated MS-2 RNA, 150 µg crude initiation factors, 40 µg tRNA, 150 µg ribosomes, 0.3 volumes S-100, and 50 pmoles N-formyl-(^{3}H)-methionyl-$tRNA_f$ were incubated at 34 C. After 3 minutes a portion was shifted to 5 C. Samples (3 µl) were taken at regular intervals from both the 34 and 5 C incubated reactions and added

to 2 mls of ice cold TCA which contained 50 µg of bovine serum albumin. The samples were then processed and the amount of hot TCA precipitable radioactivity determined as previously described (5).

Chemicals and reagents. Polyuridylic acid, phosphoenol pyruvate (monopotassium salt), adenosine 5'-triphosphate (Tris salt), guanosine 5' triphosphate (Tris salt, type VI), pyruvate kinase, puromycin dihydrochloride, adenylyl(3' 5') uridylyl (3' 5') guanosine, and $tRNA_f$ (N-formyl-methionine specific) were obtained from Sigma Chemical Co., St. Louis, MO. (^{3}H)-phenylalanine (18.5 Ci/mmole) and ^{3}H-labeled amino acid mix (1 m Ci/ml) were obtained from New England Corp., Boston, MA. MS-2 viral RNA was obtained from Miles Research Products, Elkhart, IN. Ultrapure sucrose was obtained from Schwartz-Mann, Orangeburg, NY. All other chemicals used were of reagent grade.

RESULTS

In vitro translation by 70 S particles which accumulate in E. coli at 5 C.

In a previous report (5) we demonstrated that at least some of the 70 S particles which accumulate in E. coli at 5 C were capable of synthesizing protein in vitro in the absence of an added source of exogenous mRNA. Those particles with this ability were termed 70 S monosomes (i.e., 70 S particles with mRNA associated with them, in contrast to free 70 S couples), and, we suggested that their accumulation was the result of low temperature inhibition of initiation of translation.

In an attempt to determine to what degree 70 S monosomes accumulated in E. coli at 5 C (relative to the total 70 S ribosome population), 70 S ribosomal particles were isolated at various times after a shift from 37 to 5 C and were tested for the ability to synthesize protein in the absence of an added source of exogenous mRNA (i.e. endogenous protein synthesis by 70 S monosomes, 5) and for their potential ability to synthesize protein (measured by rates of polyphenylalanine synthesis) as can be seen in Table I, the rate of endogenous protein synthesis by 70 S particles isolated from E. coli 8 hours after transfer to 5 C was 3 fold greater than the rate of endogenous protein synthesis by 70 S particles isolated 15 minutes after the shift to 5 C. The potential ability of these 70 S particles to synthesize protein (i.e., polyphenylalanine) increased only slightly over this same period. If 70 S monosomes had accumulated in E. coli at 5 C such that they constituted a large percentage of the 70 ribosome population, the number of ribosomes available for polyphenylalanine synthesis, and hence, the rate of polyphenylalanine synthesis (per mg 70 S particles) would have decreased precipitously since the rates of protein synthesis in these systems have been shown to be linear functions of ribosome concentration (5,22). Therefore, based on the relative rates of polyphenylalanine and endogenous protein

synthesis by the 70 S particles, we have estimated that after 8 hours at 5 C only 0.1% (or, about 1 in 900, see table 1) of the E. coli 70 S ribosome population were 70 S monosomes.

In vitro incorporation of N-formyl-(^{3}H)-methionine into protein at 5 C.

In a previous report (5) we have shown, using natural mRNA directed E. coli cell extract protein synthesizing systems, that, although polypeptide elongation was able to proceed at predicable rates, initiation of translation was blocked at 5 and 0 C. To test initiation of translation directly, experiments were done to determine if MS-2 RNA directed E. coli cell extract protein synthesizing systems were capable of incorporating radiolabeled N-formyl-methionine into protein (hot TCA precipitable material) after shifts from 34 to 5 C (see figure 1). At 34 C, labeled N-formyl-methionine was incorporated into protein at a linear rate for at least 10 minutes, whereas, when part of the reaction mixture was shifted to 5 C (after 3 minutes at 34 C) a negligible rate of incorporation of N-formyl-methionine into protein was observed (i.e., less than 1% of the 34 C rate). These results, taken in conjunction with our previously reported results (5) indicate that initiation of translation is blocked at 5 C.

The effect of low temperature on in vitro initiation complex formation.

We utilized the filter binding assay (21) to test initiation (i.e. 30 S initiation complex formation, see reference 27 for a review) on various sources of AuG (mRNA) codons at 5 C (see table 2). When MS-2 RNA was the source of the initiation codons for these studies, initiation complex formation (filter binding activity) was observed at 37 C but not at 25 or 5 C. These results, consistent with those of others (19, 28), may be attributed to increased tertiary structure of phage RNAs (in general) at temperatures below 30 C (19). Treatment with formaldehyde reduces the amount of phage RNA tertiary structure, allowing all of the initiation codons within the RNA to

be available for initiation at 30 C and below (19). When formaldehyde treated MS-2 RNA was utilized as a source of initiation codons, increased levels of initiation complex formation were observed at all temperatures. The 3 fold stimulation at 37 C (compared to non-formaldehyde treated MS-2 RNA) supported the contention that the formaldehyde treatment relieved the RNA tertiary structure and exposed more initiation sites. A 55% decrease in activity was observed at 5 C (relative to 37 or 25 C) in this system. When ApUpG was the source of initiation codons in this reaction, eliminating the possibility of any effects due to RNA tertiary structure, a slight stimulation of initiation complex formation (relative to 37 or 25 C) was observed at 5 C. Activities similar to those shown without ApUpG were observed when initiation factors or ribosomes were omitted from the reaction mixture (data not shown).

Figure 2 shows the kinetics of initiation complex formation (measured by the filter binding assay) at 37 and 5 C on formaldehyde treated MS-2 RNA and on ApUpG. The rate of initiation complex formation (i.e., pmoles filter retainable N-formyl-(^{3}H)-methionyl-tRNA$_f$ produced/minute) by formaldehyde treated MS-2 RNA directed systems, at 5 C was approximately 12% of the rate of 37 C (figure 2 A). However, the rate of ApUpG directed initiation complex formation was not inhibited at 5 C when either washed ribosomes (figure 2 B) or 30 S subunits were used (figure 3 C). These results indicate that: (i) the inhibition of formaldehyde treated MS-2 RNA directed initiation complex formation at 5 C may have been due to the effects of increased RNA tertiary structure on ribosome recognition of and (or) binding to initiation sites, and; (ii) the rate of formation of an ApUpG dependent 30 S initiation complex, stable on nitrocellulose filters, was uninhibited at 5 C.

To determine the effect of 5 C incubation on the formation of 70 S

initiation complexes, reaction mixtures, incubated at 37 or 5 C, were fixed with 1% glutaraldehyde (to stabilize them during high speed centrifugation, 6,15,27,33), and analyzed by sucrose gradient centrifugation. The results of an experiment testing formaldehyde treated MS-2 RNA directed 70 S initiation complex formation at 37 and 5 C are shown in figure 3. After 15 minutes at 37 C a broad peak of radioactivity in the 70 S region (single 70 S initiation complexes) and a relatively minor peak in the 120 S region (two or more 70 S initiation complexes per RNA molecule) were evident. In contrast to this, incubation of this system at 5 C for 15 minutes resulted in minimal accumulation label in the 70 and 120 S region. 30 S initiation complexes (i.e., peaks of radioactivity in the 30-40 S regions, 31,33) were not resolved in these gradient profiles; these complexes were either unstable in these gradients (in spite of glutaraldehyde fixation), and (or) they were partially masked by the large amounts of radioactivity, due to unbound N-formyl-(^{3}H)-methionyl-tRNA$_f$, at the top of the gradients. Similar experiments testing ApUpG directed 70 S initiation complex formation were done and the results are shown in figure 4. A predominant peak of label was evident in the 70 S region after only 5 minutes at 37 C (figure 4A-C) whereas, at 5 C (figure 4D-F) radioactivity accumulated in the 70 S region at a much slower rate. Calculations based on these data (areas under peaks) indicate that the rate of formation at these 70 S initiation complexes at 5 C was approximately 1 to 3% of the rate of 37 C.

The 70 S initiation complex catalyzed formation of N-formyl-methionyl-puromycin can be used as a measure of the ability of 70 S initiation complexes to catalyze peptide bond formation and, thus as a measure of the number of complete 70 S initiation complexes in the system (23). The abilities of E. coli initiation complexes, formed at either 37 or 5 C, to catalyze the formation of N-formyl-(^{3}H)-methionyl-puromycin at 37 and 5 C

are shown in figure 5. When puromycin was added to initiation complexes which were formed at 37 C and then a portion shifted to 5 C, the rates of N-formyl-(^{3}H)-methionyl-puromycin formation were identical at 37 and 5 C. However, initiation complexes which were formed at 5 C had a much lower capacity to synthesize N-formyl-(^{3}H)-methionyl-puromycin at 5 C than those which were formed at 37 C. The following conclusions were drawn from this experiment: (i) Significantly more complete and active 70 S initiation complexes were formed in 15 minutes at 37 C than at 5 C; (ii) The rate of peptide bond formation, as measured by the rates of N-formyl-(3H)-methionyl-puromycin formation, was not inhibited at 5 C, and; (iii) 70 S initiation complexes, which were formed at 37 C, did not lose their ability to catalyze the formation of N-formyl-(^{3}H)-methionyl-puromycin when shifted to 5 C.

DISCUSSION

In a previous report (5) we investigated the effects of low temperature on *in vivo* and *in vitro* translation by *E. coli* and *P. fluorescens* and demonstrated that, after shifts to 5 C, initiation of translation was inhibited in *E. coli* (but not in *P. fluorescens*) resulting in polysome runoff and the accumulation of 70 S ribosomes, some of which were 70 S monosomes. These results were supported by the results of other studies (1,6,7,11,12,28).

In the present communication, we have tested initiation of translation directly and have demonstrated that although the rate of formation of a 30 S initiation complex (detected on nitrocellulose filters) was uninhibited at 5 C, the formation of 70 S initiation complexes was inhibited to a great extent at 5 C. The formation of an active 70 S initiation complex (i.e., capable of reacting with puromycin) depends on the formation of the proper 30 S initiation complex, which contains GTP, an AUG (mRNA) codon, N-formyl-methionyl-$tRNA_f$, a 30 S subunit, IF-1 and IF-2 (see reference 23 for a review). IF-3, which binds to 30 S subunits to the same extent at 37 and 0 C (31), is released upon attachment of the N-formyl-methionyl-$tRNA_f$ to ternary 30 S complexes (32). Labile 30 S complexes have been detected in the absence of all initiation factors (30 S : MS-2 complex, 29) and in the absence of IF-1 and IF-2 (IF-3: 30 S: MS-2RNA complex, 32) at 0 C. Studies are now underway in our laboratory to determine the composition of the 30 S complexes which are formed and the fraction of these which are able to interact with 50 S ribosomal subunits to form active 70 S initiation complexes at 5 C.

The *in vitro* initiation studies also demonstrated that the rate of formation of 70 S initiation complexes, capable of reacting with puromycin, was inhibited to a great extent at 5 C, and may be the rate limiting step

in translation at 5 C. During the formation of active 70 S initiation complexes at 37 C, the joining of the 50 S ribosomal subunit to the 30 S complex, in the presence of IF-1 and IF-2, triggers GTP hydrolysis and the release of IF-1 and IF-2 (3,4,9). In the resulting complex, N-formyl-methionyl-tRNA is located at a site which corresponds to the post-translocation stage (2,20). In the absence of IF-1 or GTP hydrolysis, IF-2 release does not occur, leading to the formation of "abortive" initiation complexes which are unable to function in protein synthesis (3,4). Although GTP stimulates the rate of N-formyl-methionyl-puromycin formation (14), the energy derived from GTP hydrolysis does not appear to be required for 30 S initiation complexes, in the presence of 50 S subunits, to react with puromycin (10). Rather, it appears that the role of GTP in initiation of translation is to increase the rate of initiation complex formation by increasing the affinity of IF-2 for 30 S ribosomes upon GTP hydrolysis, IF-2 is released from the initiation complex enabling N-formyl-methionyl-tRNA to interact with puromycin (2,10). Investigations are now underway in our laboratory to determine the relative efficiencies of these interactions at low temperature.

The experiments in which we determined the rates of endogenous protein synthesis and polyphenylalanine synthesis, by the 70 S ribosomes that accumulated at 5 C, indicate that 70 S monosomes were only a small fraction of the population of 70 S ribosomes that accumulated in E. coli at 5 C. This is supported by previous reports which demonstrated that most of the 70 S ribosomes, isolated from E. coli after a shift from 37 to 5 C, were susceptible to high speed centrifugation-induced dissociation (5,6,11,15) and by the fact that the rate of formation of 70 S initiation complexes was inhibited at 5 C (see data and discussion above). These data support the contention that the 70 S monosomes accumulated as a result of a partial

(but not complete) block of initiation of translation. It seems reasonable to conclude that the small population of 70 S monosomes that accumulated in E. coli at 5 C were 70 S post-initiation monosomes (i.e., 70 S monosomes that had successfully completed initiation of translation and were completing the polypeptide chains coded for by their nascent mRNAs at the time of their isolation) for the following reasons: (i) Low temperature did not appear to cause a complete block of any step of initiation of translation (e.g., even 70 S initiation complex formation was able to proceed at a diminished rate at 5 C; (ii) The 70 S monosomes were capable of in vitro translation at temperatures ranging from 37 to 5 C (see reference 5), and; (iii) Polypeptide elongation has been shown to proceed at predictable (from Arrhenius plots) rates at 5 C (5,7,28). This implies that the 70 S monosome population per se was a heterogeneous population of active monosomes at different points along their respective mRNAs, each containing nascent polypeptides at various stages of completion. Net rates of in vivo and in vitro polypeptide synthesis by such a small percentage of the total ribosome population would be expected to be low and therefore, difficult to detect.

At the present time we cannot exclude the possibility that the mRNAs associated with the monosomes were susceptible to endonucleolytic cleavage (1) and therefore, were unable to synthesize complete polypeptides (in vivo or in vitro) as efficiently as ribosomes in the polysome configuration. However, it may be that the integrity of the mRNA, which was associated with the 70 S monosome, was protected by the ribosome (24) or by increased mRNA stability (13,16) at 5 C.

Ruscetti and Jacobson (24) demonstrated that 70 S monosomes (i.e., 70 S ribosomes bound near one end of the mRNA strands, 17) accumulated in E. coli after a nutritional shift down. However, it was unclear what percentage of the total 70 S ribosome population were 70 S monosomes. It was suggested

that an inhibition at the level of initiation of translation, which resulted in an increased "dwell time" of newly bound ribosomes at initiation sites, was the reason for 70 S monosome accumulation under these conditions.

A general scheme for the effects of a low temperature shift of the E. coli translation system emerges from the results presented in this paper and from the data which has accumulated in the literature (see figure 6). Shifting E. coli from 37 C to low temperature results in polysome runoff and the accumulation of 70 S ribosomes. This is supported by the low temperature previously reported results (5,6,7) and by the model for the ribosome cycle proposed by Davis (8). The 70 S ribosomes which accumulate at 5 C are in equilibrium with free ribosomal subunits (reaction 2). This is also supported by the polysome profiles from low temperature incubated E. coli presented by others (6,7,11). IF-3 is able to interact with 30 S subunits (reaction 3) at temperatures as low as 0 C (31), leading to the formation of "native subunits" (8,12). However, the majority of evidence indicates that the equilibrium (reactions 2 and 4) of the ribosome population at low temperature is shifted toward free 70 S ribosomes (sensitive to dissociation during high speed centrifugation (5,6,7,26). The rate of formation of 30 S initiation complexes is uninhibited at low temperature. However, although the formation of these complexes at 5 C is dependent on the presence of N-formyl-methionyl-$tRNA_f$, initiation factors, ribosomes, and a source of initiation (AUG) codons, and, although 70 S initiation complexes are formed at a slow rate at 5 C (see data above), it is not clear at the present time whether the rate of "active" 30 S initiation complex formation is inhibited at low temperature (i.e., reactions 5 + 6). Therefore, this model predicts that initiation proceeds normally at low temperature, at least through reaction 5. Since the rate of formation of 70 S initiation complexes, able to react with puromycin, proceeds very slowly at 5 C, this model proposes two possible

sites for the low temperature inhibition of translation: (i) an inhibition of the rate of formation of "active" 30 S initiation complexes from 30 S initiation complexes intermediates (i.e., reaction 6), or; (ii) decreased abilities of IF-1, IF-2, GTP (hydrolysis), (and) or 50 S subunits to interact properly with 30 S initiation complexes to form 70 S initiation complexes (monosomes, i.e., reaction 7). The fact that some 70 S initiation complexes, able to react with puromycin, form at low temperature (see figure 2-5), indicates that initiation is not completely blocked at low temperature, and allows for the formation of 70 S initiation complexes (monosomes). Particles which successfully complete initiation at 5 C (reactions 5-6) are able to complete rounds of polypeptide elongation (reaction 9) at low temperature (5) due to the fact that elongation is not inhibited to the extent that initiation is inhibited at low temperature (1, 5,7,12,28). Upon release of the complete (or incomplete, if mRNA degradation occurs,) polypeptide, the released ribosomes return (by reaction 10) to the (low temperature equilibrated) ribosome pool (3).

LITERATURE CITED

1. Anderson, W. A. 1975. Kinetics of β-galactosidase synthesis in Escherichia coli at 5 C. J. Bacteriol. 121: 907-916.

2. Benne, R., and H. O. Voorma. 1972. Entry site of formyl-methionyl-tRNA. FEBS Lett. 47: 447-450.

3. Benne, R., F. Ebes, and H. O. Voorma. 1973. Sequence of events in initiation of protein synthesis. Eur. J. Biochem. 38: 265-273.

4. Benne, R., H. Naaktgeboren, J. Gubbens, and H. O. Voorma. 1973. Recycling of initiation factors IF-1, IF-2, and IF-3. Eur. J. Biochem. 32: 372-380.

5. Breeze, R. J., C. J. Solomon, and D. H. Pope. 1978. The effects of low temperature on in vivo and in vitro protein synthesis by Escherichia coli and Pseudomonas fluorescens. J. Bacteriol. 134: 861-874.

6. Chlamovitch, Y. P., and W. A. Anderson. 1972. Run off ribosomes of Escherichia coli: their accumulation in response to cold treatment and their dissociation during centrifugation. FEBS Lett. 23: 83-86.

7. Das, H. K. and A. Goldstein. 1968. Limited capacity for protein synthesis at zero degrees centigrade in Escherichia coli. J. Mol. Biol. 31: 209-216.

8. Davis, B. D. 1974. Alternate views on the ribosome cycle. In M. Nomura, A. Tissieres, and P. Lengyel (eds.), Ribosomes, pp. 705-710. Cold Spring Harbor Press, Cold Spring Harbor, NY.

9. Dubnoff, J. S. and U. Maitra. 1972. Characterization of the ribosome-dependent guanosine triphosphatase activity of polypeptide chain initiation factor IF-2. J. Biol. Chem. 247: 2876-2883.

10. Dubnoff, J. S., A. H. Lockwood, and U. Maitra. 1972. Studies on the role of guanosine triphosphate in polypeptide chain initiation in Escherichia coli. J. Biol. Chem. 247: 2884-2894.

11. Friedman, H., P. Lu, and A. Rich. 1969. Ribosomal subunits produced by cold sensitive initiation of protein synthesis. Nature 223: 909-913.

12. Friedman, H., P. Lu, and A. Rich. 1971. Temperature control of initiation of protein synthesis in Escherichia coli. J. Mol. Biol. 61: 105-121.

13. Friesen, J. D. 1968. Control of messenger RNA synthesis and decay in Escherichia coli. J. Mol. Biol. 20: 559-573.

14. Hershey, J. W. B. and R. E. Thach. 1967. Role of guanosine 5'-triphosphate in the formation of peptide initiation, I. Synthesis of formyl-methionyl-puromycin. Proc. Natl. Acad. Sci. U.S.A. 57: 759-766.

15. Infante, A. A. and R. Baierlei . 1971. Pressure-induced dissociation of sedimenting ribosomes: effect on sedimentation patterns. Proc. Natl. Acad. Sci. U.S.A. 68: 1780-1785.

16. Jacobson, L. A. 1970. Regulation of ribonucleic acid synthesis in Escherichia coli during diauxic lag: accumulation of heterogeneous ribonucleic acid. J. Bacteriol. 102: 740-746.

17. Jacobson, L. A. and J. C. Baldassare. 1976. Association of messenger ribonucleic acid with 70 S monosomes from down shifted Escherichia coli. J. Bacteriol.

18. Leder, P. and H. Bursztyn. 1966. Initiation of protein synthesis, II. A convenient assay for the ribosome-dependent synthesis of N-formyl-C^{14}-methionyl-puromycin. Biochem. Biophys. Res. Comm. 25: 233-238.

19. Lodish, H. F. 1975. Regulation of in vitro protein synthesis by bacteriophage RNA by RNA tertiary structure. In N. D. Zinder (ed.), RNA phages., pp. 319-352. Cold Spring Harbor Press, Cold Spring Harbor, NY.

20. McEwen, C. R. 1967. Tables for estimating sedimentation through linear concentration gradients of sucrose solution. Analytical Biochem. 20: 114-149.

21. Nirenberg, M. and P. Leder. 1964. RNA codewords and protein synthesis. Science 145: 1399-1407.

22. Pope, D. H., W. P. Smith, R. W. Swartz, and J. V. Landau. 1975. Role of bacterial ribosomes in barotolerance. J. Bacteriol. 121: 664-669.

23. Revel, M. 1977. Initiation of messenger RNA translation into protein and some aspects of its regulation. In H. Weissbach and S. Pestka (eds.), Molecular mechanisms of protein biosynthesis, pp. 246-321. Academic Press, New York.

24. Ruscetti, F. W. and L. A. Jacobson. 1972. Accumulation of 70 S monoribosomes in Escherichia coli after an energy source shift down. J. Bacteriol. 111: 142-151.

25. Shaw, M. K., A. C. Murr, and J. L. Ingraham. 1971. Determination of the minimal temperature for growth of Escherichia coli. J. Bacteriol. 105: 683-684.

26. Spirin, A. S. 1972. Association between ribosomal subparticles and its functional significance. FEBS Symposium, Volume 23, pp. 197-228. Academic Press, London.

27. Subramanian, A. R. 1972. Glutaraldehyde fixation of ribosomes. Its use in the analysis of ribosome dissociation. Biochemistry 11: 2710-2714.

28. Szer, W. 1970. Cell free protein synthesis at 0 C. An activating factor from ribosomes from a psychrophilic organism. Biochim. Biophys. Acta 213: 159-170.

29. Szer, W. and S. Leffler. 1974. Interaction of Escherichia coli 30 S ribosomal subunits with MS-2 phage RNA in the absence of initiation factors. Proc. Natl. Acad. Sci, U.S.A. 71: 3611-3615.

30. Thach, S. and R. E. Thach. 1971. Translocation of messenger RNA and "accommodation" of fmet-tRNA. Proc. Natl. Acad. Sci. U.S.A. 68: 1791-1795.

31. Vermeer, C., J. Boon, A. Talens, and L. Bosch. 1973. Binding of the initiation factor IF-3 to Escherichia coli ribosomes and MS-2 RNA. Eur. J. Biochem. 40: 283-293.

32. Vermeer, C., W. Van Alphen, P. Van Knippenberg, and L Bosch. 1973. Initiation factor dependent binding of MS-2 RNA to 30 S ribosomes and the recycling of IF-3. Eur. J. Biochem. 40: 295-308.

33. Waterson, J., N. L. Sopori, S. L. Gupta, P. Lengyel. 1972. Apparent changes in ribosomes conformation during protein biosynthesis. Centrifugation at high speed distorts initiation, pre-translocation and post-translocation complexes to a different extent. Biochemistry 11: 1377-1382.

Table 1. Rates of amino acid incorporation into protein by 70 S ribosomes which were isolated from E. coli at various times after a shift from 37 to 5 C.

Time of isolation of 70 S particles (hours after the shift to 5 C)	pmoles amino acids incorporated into protein/ min./mg 70 S particles[a]. endogenous mRNA directed	poly U directed	Ratio of poly-U: endogenous rates
0.25	0.17	430.1	2530 : 1
1	0.20	445.1	2229 : 1
3	0.54	486.0	900 : 1

a. The rates of amino acid incorporation into protein in the absence of an added source of mRNA, and of poly-U directed incorporation of phenylalanine into polyphenylalanine by 70 S particles at 25 C were determined as described in Materials and Methods.

Table 2. The effect of temperature on the ApUpG, MS-2 RNA, and HCHO-MS-2 RNA[a] directed binding of N-formyl-(^{3}H)-methionyl-tRNA$_f$ to ribosomes; filter binding assay.

Source of initiation codon	pmoles N-formyl-(^{3}H)-methionyl-tRNA retained on nitrocellulose filters/A_{260} unit ribosomes[b].		
	37 C	25 C	5 C
MS-2 RNA	2.36 (0.12)[c]	0.28 (0.12)	0.15 (0.12)
HCHO-MS-2 RNA	7.55 (0.41)	7.45 (0.39)	3.21 (0.40)
ApUpG	6.33 (0.39)	6.36 (0.32)	8.03 (0.29)

a. Formaldehyde treated MS-2 RNA.

b. 50 µl reaction mixtures (described in Materials and Methods) containing either MS-2 RNA, formaldehyde treated MS-2 RNA, or ApUpG as the source of initiation codon were incubated at the indicated temperatures for 15 minutes, added to 2 mls of ice cold buffer A, and filtered on nitrocellulose filters. The filters were washed twice with ice cold buffer A and the amount of N-formyl-(^{3}H)-methionyl-tRNA$_f$ retained on the filters determined.

c. The values in the parentheses are those obtained when the initiation codon source was omitted from the reaction mixture.

FIGURE LEGENDS

Figure 1. Incorporation of N-formyl-(^{3}H)-methionine into protein at 34 and 5 C. Reaction mixtures (100 µl, described in Materials and Methods) were incubated at 34 C, and, after 3 minutes, a portion of the reaction mixture was shifted to 5 C. Samples (5 ml) were taken at the indicated times from the 34 C (•) and 5 C (○) incubated reaction mixtures and the amount of N-formyl-(^{3}H)-methionine incorporated into protein determined as described in Materials and Methods.

Figure 2. Kinetics of formaldehyde treated MS-2 RNA or ApUpG directed initiation complex formation at 37 and 5 C. Reaction mixtures (50 µl) which contained 50 pmoles f-formyl-(^{3}H)-methionyl-tRNA$_f$, 0.2 µmoles Tris-GTP, 150 µg crude initiation factors, 2% glycerol, 50 mM Tris-HCl pH 7.6, 150 mM NH_4Cl, 3.5 mM $Mg(OAc)_2$, 6 mM 2-mercaptoethanol, 1 A_{260} units ribosomes (panels A & B) or 0.5 A_{260} units 30 S subunits (panel C) and 20 µg formaldehyde treated MS-2 RNA (panel A) or 0.05 A_{260} units ApUpG (panels B & C) were incubated at 37 or 5 C. At the times indicated samples (5 ml) were added to 2 ml ice cold buffer A, filtered onto nitrocellulose filters and the amount of filter-bound N-formyl-(^{3}H)-methionyl-tRNA$_f$ retained on filters determined as described in Materials and Methods. Symbols: 37 C (●); 5 C (○); control reaction mixture, were incubated in the absence of ApUpG at 37 (▲) or 5 (△).

Figure 3. Sucrose gradient analysis of formaldehyde treated MS-2 RNA directed initiation complex formation at 37 and 5 C. Reaction mixtures (100 µl), similar to those described in the legend for figure 2, panel A, were incubated at 37 C for 0 minutes (panel A) and 15 minutes (panel B) or at 5 C for 1 hour (panel C). They were then fixed with glutaraldehyde, layered on 15-30% sucrose gradients, and centrifuged at 40,000 rpm for 6 hours in a SW 41 rotor. The contents of each gradients were monitored at 260 nm, fractionated, and the radioactivity in each fraction determined as described in Materials and Methods.

Figure 4. Sucrose gradient analysis of ApUpG directed initiation complex formation at 37 and 5 C. Reaction mixtures (100 µl) similar to those described in the legend for figure 2, panel B, were incubated at 37 C for 0 minutes (panel A), 5 minutes (panel B) and 15 minutes (panel C) and at 5 C for 0 minutes (panel D), 15 minutes (panel E) and 60 minutes (panel F). They were then fixed with glutaraldehyde and processed as described in the legend for figure 3.

Figure 5. Synthesis of N-formyl-(^{3}H)-methionyl-puromycin by initiation complexes formed at 37 and 5 C. Initiation complexes were prepared by incubating reaction mixtures, identical to those described in the legend for figure 2 panel B, at 37 C for 15 minutes. After chilling on ice for 2 minutes, puromycin 60 µM) was added to each and the reactions were returned 37 (●) or 5 C (○). At the times indicated samples (8 µl) were added to 1 ml of 0.1 N

sodium acetate pH 5.5 and the amount of N-formyl-(3H)-methionyl-puromycin determined as described in Materials and Methods. A similar experiment was done in which initiation complexes were prepared (i.e. reaction mixtures incubated for 15 minutes) at 5 C, chilled, puromycin added, the reaction returned to 5 C, and N-formyl-(3H)-methionyl-puromycin formation followed as described above (Δ).

Figure 6. A general scheme which depicts the effect of a low temperature shift on the E. coli translation system.

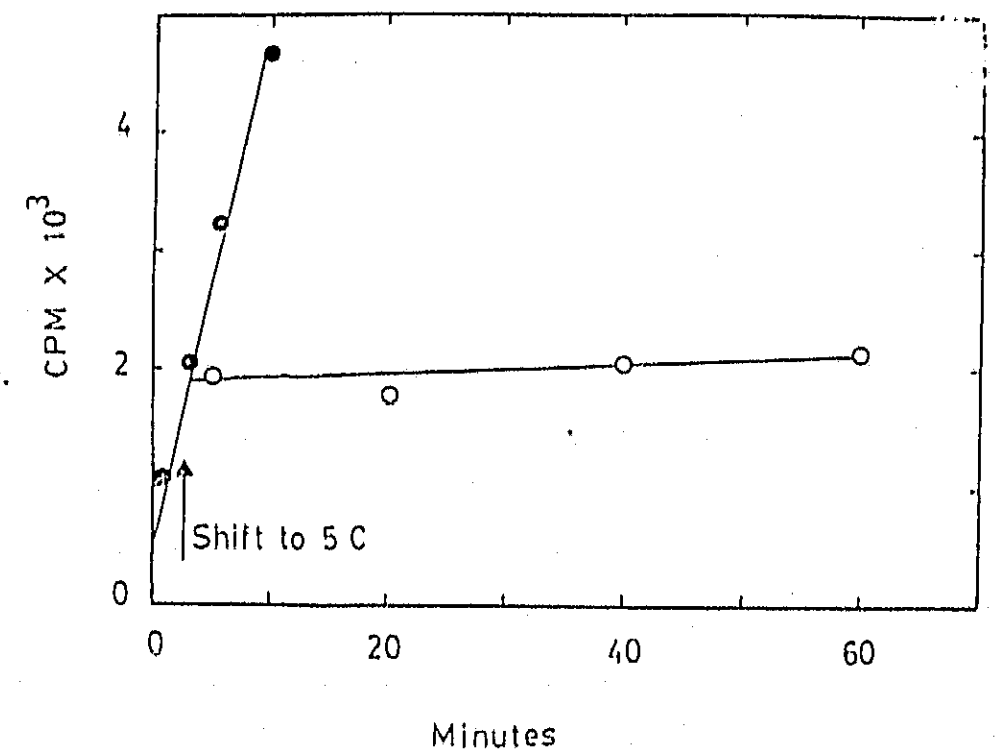

Figure 1.

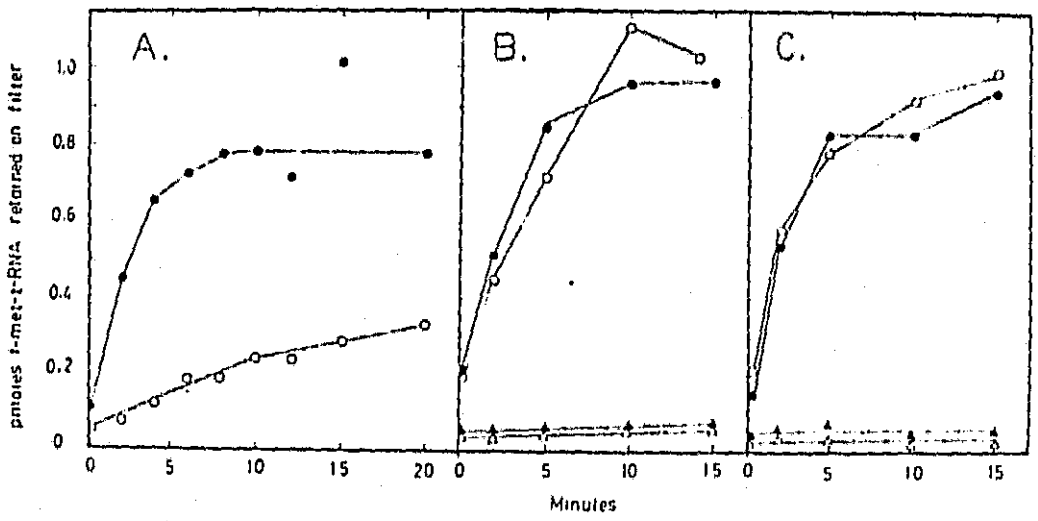

Figure 2.

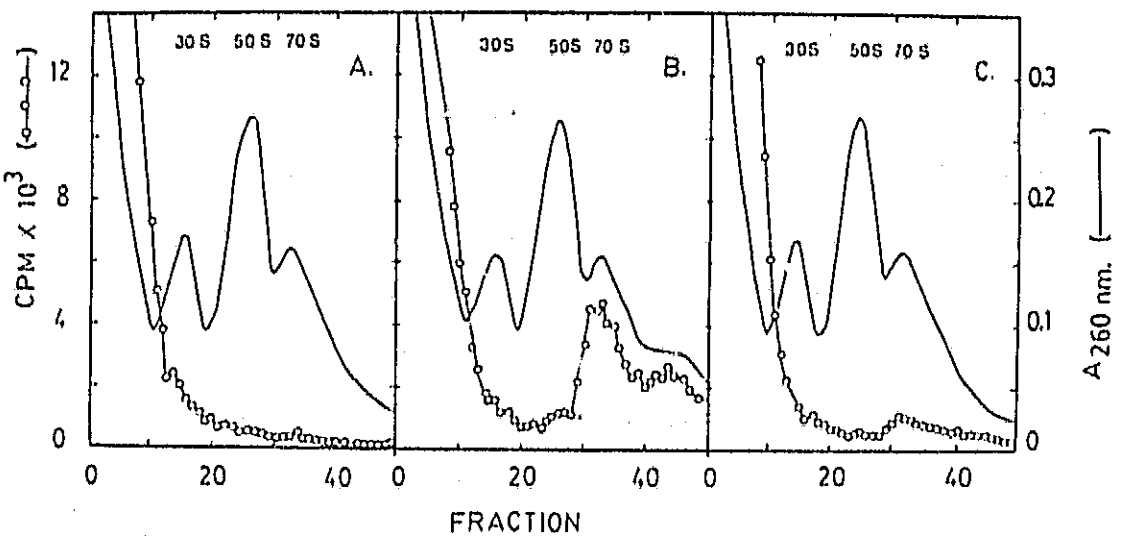

Figure 3.

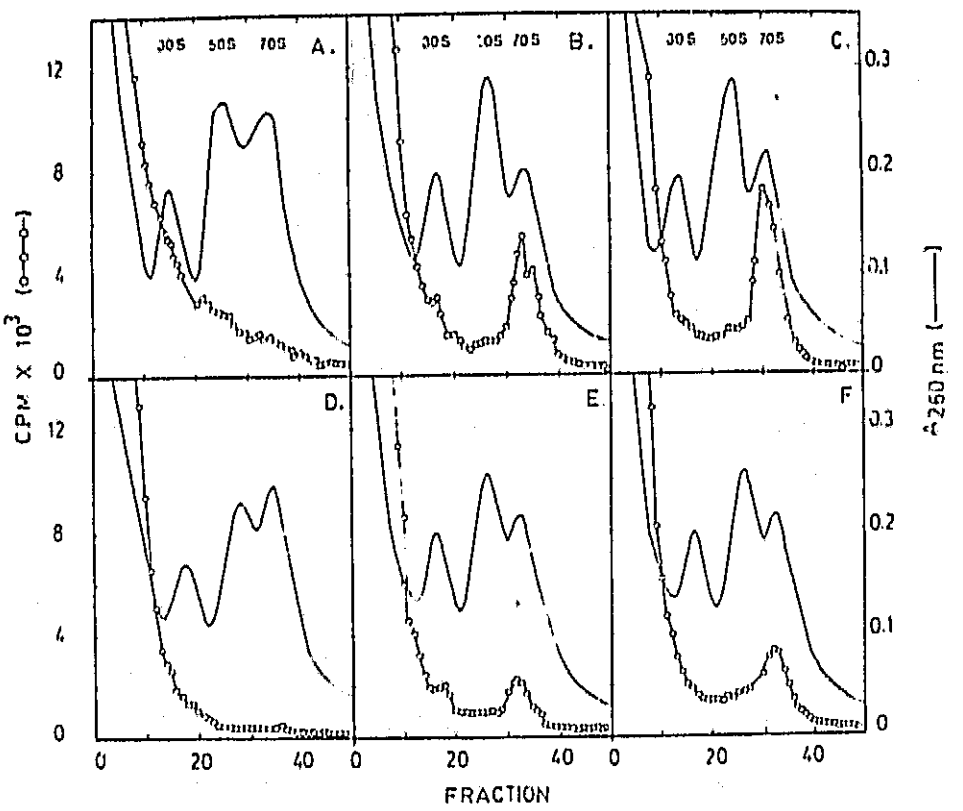

Figure 4.

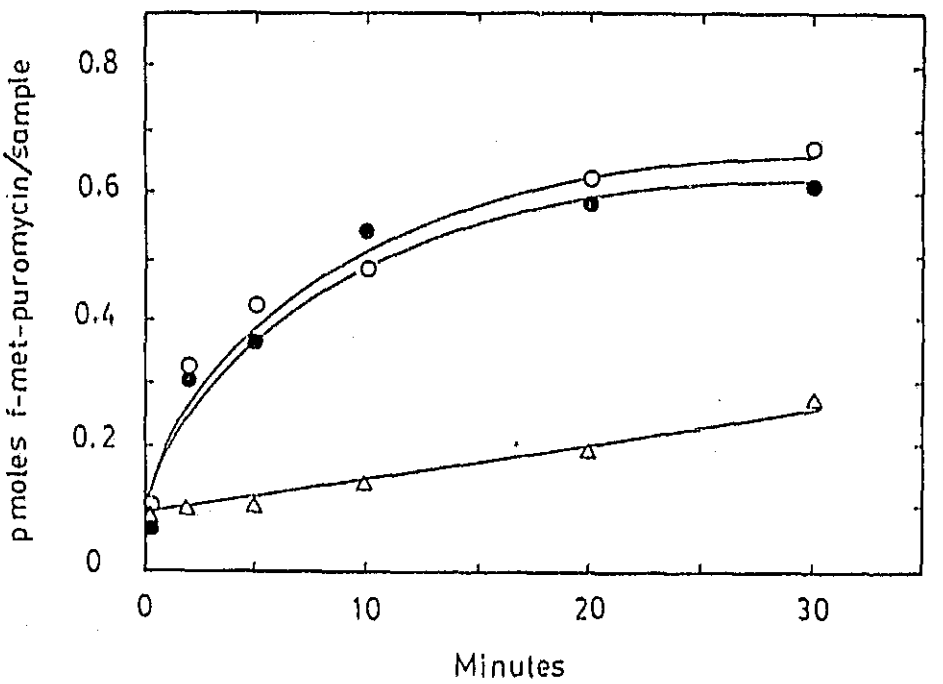

Figure 5.

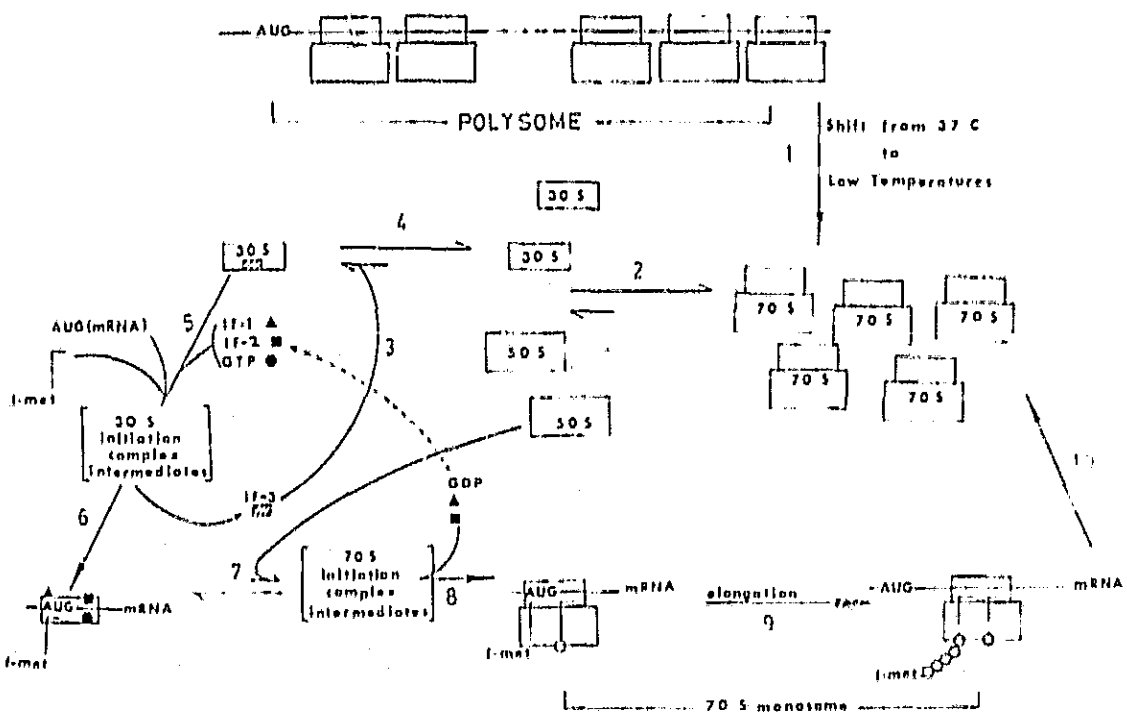

Figure 6